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(54) Title: METHODS OF INCREASING RATES OF INFECTION BY DIRECTING MOTION OF VECTORS**(57) Abstract**

This invention provides methods of increasing the frequency of contact between vectors and stationary target cells in an apparatus containing them which involves causing the vectors to move towards the target cells with motion above and beyond random Brownian motion. The methods of this invention include causing the vectors to move in the direction of the cells by (1) causing flow-through of a liquid containing the vectors through or past a cell bed, (2) moving charged vectors towards the target cells by electrodiffusion and (3) centrifuging vectors and cells to cause settling of vectors onto the cells.

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METHODS OF INCREASING RATES OF INFECTION
BY DIRECTING MOTION OF VECTORS

BACKGROUND OF THE INVENTION

This invention relates generally to genetic
5 engineering, and more specifically, to methods of
increasing infection efficiency.

Scientists now routinely introduce genetic
material into prokaryotic or eukaryotic cells to obtain
the expression of heterologous genes. The application of
10 this technology to human cells forms the basis for the
burgeoning field of gene therapy. Typical methods
include incorporating DNA directly into cells, for
example, by calcium phosphate precipitation, and using
vector-mediated infection. Scientists have used a wide
15 variety of viruses as gene-transfer vectors. In
particular, non-competent retroviruses have proved useful
because they allow the stable introduction of genetic
material into the chromosome of the infected target cell.

The process of retroviral infection involves
20 many steps. In the initial step the virus comes in
contact with the cell. Next, the viral RNA is
internalized. Reverse transcription follows. Then the
viral DNA enters the nucleus. Finally the virally
derived DNA is integrated into the target cell's
25 chromosomes. This step is believed to require cell
division.

Retroviruses are assembled by so-called
packaging cell lines. Scientists have developed two
methods to infect a target cell with a retrovirus.
30 First, one may co-culture the target cell with the
packaging cell line. This approach has proven
successful, but unfortunately it represents the
clinically less attractive procedure. There is the risk

of contaminating the infected target cells with cells from the packaging line, with subsequent risks.

Second, one may culture a bed of target cells and add to them spent medium from the packaging cell line. Scientists believe that this approach is limited by the concentration of the virus in the spent medium. It has proven difficult to obtain high titer supernatants from packaging cell lines because the viruses tends to disintegrate rapidly. In any case, attempts to increase the concentration of the virus has not led yet to a dramatic increase in rates of infection.

Thus, there exists a need for methods to increase the rate of infection of target cells by retroviral vectors. The present invention satisfies this need and provides related advantages as well by providing methods to direct to motion of vectors towards the target cells.

SUMMARY OF THE INVENTION

This invention provides a method of increasing the frequency of contact between vectors and stationary target cells in an apparatus containing them by causing the vectors to move towards the target cells over and above the movement caused by random Brownian motion.

This invention also provides the method of moving the vectors towards the target cells in which the vectors are contained in a liquid which is moved towards the target cells. In one embodiment of this invention, the apparatus includes a container having a porous surface which supports the target cells, wherein the container contains the liquid in contact with the porous surface, and the liquid is caused to move through the porous surface.

In another embodiment of this method, the apparatus further includes a centrifuge for spinning the container, and the method involves allowing the liquid to move through the porous membrane under the force of enhanced gravity created by spinning the container in the centrifuge.

In another embodiment of this method, the apparatus includes target cells plated on the face of a spinable disk, and the method involves causing the vectors to move towards the cells by spinning the disk around an axis of rotation essentially perpendicular to the face of the disk, and directing a stream of the liquid towards the cells and substantially along the axis of rotation.

This invention also provides the method of causing the vectors to move towards the target cells by applying electrostatic force to the vectors. In one embodiment, the apparatus comprises an electrophoresis unit having first and second chambers separated by an ion-permeable membrane, the first chamber having an electrolytic fluid and a negative electrode in contact therewith, the second chamber having an electrolytic liquid and a positive electrode in contact therewith; and means for applying voltage across the positive and negative electrodes. The apparatus contains negatively charged vectors and target cells plated on the surface of the ion-permeable membrane in the first chamber.

This invention also provides the method of causing the vectors to move towards the cells by applying elevated gravitational force to induce sedimentation of the virus onto the target cells. In one embodiment, the apparatus includes a centrifuge having a rotor which has a surface which supports the target cells under centrifugal force and a liquid containing the vectors in contact with the surface.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts an embodiment of this invention in which a liquid infection medium containing the vectors are moved past the target cell bed and
5 through a porous membrane by convective flow.

Figure 2 depicts an embodiment of this invention in which liquid infection medium flows past the target cell bed and through the porous membrane by force of gravity. The container in this Figure is a cell
10 culture insert.

Figure 3 depicts an embodiment of this invention in which liquid infection medium is recycled into the container. A pump draws medium that has already flowed past the cells into a reservoir and pumps the
15 liquid back into the container.

Figure 4 depicts an embodiment of this invention in which a liquid containing the vector is moved past the target cells by flow-through using positive pressure. A syringe pump connected to the
20 container above the liquid supplies the pressure.

Figure 5 depicts an embodiment of this invention in which a liquid containing the vector is moved past the target cells by flow-through using negative pressure. An aspirator connected to the
25 container below the liquid supplies the suction.

Figure 6 depicts an embodiment of this invention in which the liquid infection medium is caused to flow past the target cell bed by enhanced gravity. The container is placed in the rotor of a centrifuge and
30 spun around an axis in the direction of the arrow.

Figure 7 depicts an embodiment of this invention involving a spinning disk. The disk on which the target cell bed is plated spins around an axis of rotation. Liquid infection medium is directed toward the surface of the disk as indicated by the heavy arrow. The liquid spreads out over the surface of the disk and comes into contact with the cell bed as indicated by the light arrows.

Figure 8 depicts an embodiment of this invention in which the vectors are moved towards the target cells by electrodifusion. An electrophoresis chamber includes a container with a permeable membrane on which the target cells are plated includes the liquid infection medium and a negative electrode. The chamber also includes a positive electrode in contact with electrophoresis buffer on the other side of the membrane. A power supply supplies the electrostatic force.

Figure 9 depicts an embodiment of this invention in which the vectors are caused to move towards the target cells by means of centrifugal force in a centrifuge.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides methods that dramatically improve the infection efficiency of target cells by a vector by significantly increasing the number of contacts between vectors and target cells in a infection system. These methods are particularly useful for increasing infection rates by vectors having short half-lives, such as retroviruses. In a infection system comprising murine amphotropic virus and CV-1 cells, results demonstrate infection rates 10 to 30 times greater than that obtained using currently available methods.

This invention results from the application of a physico-chemical approach to the problem of cell infection. Infection requires physical contact between a vector and a target cell. Contact, in turn, is limited by two processes: the diffusion of the vector and the degradation of the vector. Each process has a characteristic time constant from which one can determine its rate.

The average time for a vector to travel a particular distance in one dimension is given by the classical Einstein equation for Brownian motion. (See, for example, E.L. Cussler, Diffusion, Mass Transfer in Fluid Systems, (1984)):

$$t_{\text{diff}} = l^2/2D \quad (1)$$

where l is the diffusional distance in question. In a system including a bed of target cells covered by a layer of liquid containing the vectors, the diffusional distance is the depth of the liquid layer containing the vectors which covers the target cells. D is the diffusion coefficient. The numerical value of the diffusion constant for a vector can be estimated from the Stokes-Einstein equation:

$$D = \frac{k_B T}{6\pi\mu R_0} \quad (2)$$

where k_B is the Boltzmann constant ($1.38 \cdot 10^{-16} \text{ erg/}^\circ\text{K}$); $T = 310^\circ\text{K}$, μ is the medium viscosity (about 1 cP for water and cell culture medium) and R_0 is hydrodynamic radius of the virus. For murine amphotropic virus R_0 is about 0.05 microns. Substituting these numerical values into the above equation one obtains $D = 4.5 \cdot 10^{-8} \text{ cm}^2/\text{sec}$. This value is about one one-hundredth of typical values for small organic molecules, such as glucose, and about one

one-tenth of that observed for proteins. Thus, the average time for a murine amphotropic virus to diffuse 3 mm to the cell bed is

$$t_{diff} = l^2/2D = 10^6 \text{ seconds} \quad (3)$$

5 or about 11.5 days.

The half-life ($t_{0.5}$) of retroviruses is short. The half-life for the retrovirus produced by the CRIP cell line, used for the experiments reported below, has been measured as 5.5 hours. B.A. Shen et al., "Kinetics of Retroviral Production from the Amphotropic ψ CRIP Murine Producer Cell Line," Biotech & Bioeng., (in press).

Based on the diffusion constant and the decay time, one can estimate the distance that an average viral vector can travel before one half-life has passed:

$$15 \quad l_{0.5} = \sqrt{2D \cdot t_{0.5}} = 400 \text{ microns} \quad (5)$$

This distance is short, compared to the usual depths of liquid that are used in standard infection protocols. In fact, if one uses four half-lives in the above equation, one finds out that over 93% of the viruses have fallen apart after diffusing only 800 microns. Thus, in a typical infection procedure, in which the liquid layer is about 3000 microns (i.e., 3 mm) those virions beyond about 500 to 800 microns from the target cell have small probability of ever encountering the cell.

Thus, diffusion limits the average distance a vector can travel in solution in a given period of time. For vectors with short half-lives, such as retroviruses,

diffusion limits the absolute number of contacts between vectors and target cells in a system comprising them.

This invention overcomes the diffusion-imposed limit on the rate and number of contacts between vectors and target cells by causing the vectors to move towards stationary target cells in an apparatus containing them, that is, by imparting a directed, biased or non-random motion to the vectors in the direction of the target cells. As a result of moving the vectors towards cells, they acquire a positive net velocity in the direction of the target cells in contrast to the zero net velocity of the vectors in solution due to Brownian motion.

The methods of this invention include causing the vectors to move in the direction of the cells by (1) causing flow-through of a liquid containing the vectors through or past a cell bed, (2) moving charged vectors towards the target cells by electrodifussion and (3) centrifuging vectors and cells to cause settling of vectors onto the cells.

As used herein, the term "vector" means any particle capable of transferring a gene to a target cell in the infection process. Vectors known to the art include, for example, viruses, spheroplasts or liposomes containing genes, and free nucleic acids containing genes, such as plasmids or nucleic acid fragments.

Viruses useful in the methods of this invention include retroviruses (such as murine amphotropic virus), baculovirus, SV40-type viruses, polyoma viruses, adenoviruses, Epstein-Barr viruses, herpes simplex virus, vaccinia viruses and papilloma viruses. M. Krieger, Gene Transfer and Expression: A Laboratory Manual, W.H. Freeman and Company, New York, New York (1990) and Methods in Enzymology, vol. 185, articles 38-44 (D.V.

Goeddel, ed.) (1990), both incorporated herein by reference, further describe these vectors and their use. One can readily employ other vectors not named but known to the art.

5 The methods of this invention are particularly useful for increasing rates of infection by vectors having half-lives of less than 24 hours, such as the murine amphotropic virus and baculovirus. However, they are also useful for increasing rates of infection for
10 vectors having longer half-lives. Adsorption of vectors by cells depends, in part, on concentration of the vector in solution. By imparting directed motion to the vectors in the direction of the cells, the methods of this invention effectively increase the concentration of the
15 vectors in the vicinity of the cells, resulting in more adsorption and infection.

As used herein, the term "target cell" refers to cells supported on a surface and capable of being infected by a vector. Cells useful for gene therapy are
20 particularly useful in this invention. They include, for example, bone marrow cells, lymphocytes, fibroblasts, keratinocytes, hepatocytes, endothelial cells, neurons, muscle cells, and epithelial cells. According to one embodiment, this invention uses hematopoietic stem cells,
25 particularly of human origin. This invention further contemplates use of cells taken from a patient or subject with the intent of infecting those cells and re-introducing them into the patient or subject.

The target cells of this invention are
30 substantially stationary with respect to the surface of the apparatus that supports them. Target cells can be attached cells, such as fibroblasts or stem cells. They also can be suspension cells which have settled onto the surface.

This invention is directed to methods of causing the vectors to move towards the stationary target cells by causing the liquid containing the vectors to flow past stationary target cells, for example, past or
5 through a cell bed. Flow-through of liquid is also referred to as "convective flow" or "bulk flow."

In one embodiment of the flow-through method, the apparatus comprises a container having a porous surface which supports the target cells, and the
10 container contains the liquid in contact with the porous surface. The vectors are moved towards the target cells by causing the liquid to flow through the porous surface. Figure 1 depicts this embodiment.

It has been found that porous surfaces having
15 pores large enough to allow passage of the vector are effective in this invention. Smaller pores tend to become clogged with the vector, especially in high retroviral titer supernatants. Porous surfaces having pores small enough so that the target cells cannot pass
20 through are effective. In practice, this is a pore size range of about 0.1 microns to about 2 microns.

The effective viral motion is the average fluid velocity, given by the volumetric flow rate divided by the porous surface area. Flow rates above 0.01 cm/hr
25 overcome the viral motion due to diffusion. The flow rate also should be slow enough to avoid shearing of the cells. Effective flow rates include between about 0.01 cm/hr to about 1.0 cm/hr, and flow rates above about 0.1 cm/hr are most efficient.

30 Cells should not be exposed to flow-through so long that they have significant decrease in viability. While this time will vary depending on cell lines,

flow-through may proceed effectively for about 1 hr to about 10 hrs. It has been found that exposing CV-1 cells to flow-through for 2 to 3 hours does not decrease viability.

5 Cells usefully can be seeded at density of about 5% to about 90% of confluence on the porous surface. High density tends to block pores and decrease flow. However, this limitation is overcome by using larger pore sizes when cells are plated at higher density. After plating target cells on the porous surface, the liquid medium containing the vector is added. The liquid is then caused to flow past the cells.

In any of the methods of this invention, when cells are transfected with retroviruses at densities greater than about 50% confluency, it is preferable to transfer them after infection to lower density at which growth can occur and the retrovirus can integrate into the host genome.

This method offers the advantage that the cell bed can be oxygenated from below since the lower surface of the membrane is exposed to the atmosphere. Such oxygenation may be advantageous when one uses higher cell densities on the membrane. It may also be useful to stimulate cell division, which is necessary for retroviral integration into the target cell's DNA.

A cell culture insert of about 6 ml makes a suitable container in this embodiment. A cell culture insert is a cup having a filtration membrane as the bottom surface, constituting the porous surface.

30 TRANSWELL® (Costar, Cambridge, Massachusetts) or FALCON

cell culture inserts (Becton Dickinson, Lincoln Park, New Jersey) are useful in this invention as the container.

When using a cell culture insert as the container, the system can be placed into the well of a culture dish to allow filtration and to collect the fluid. Convective flow of the liquid can proceed by allowing it to move under the force of gravity created by hydrostatic head. In this method, the liquid infection medium is added to the container and allowed to drip past the cells and through the porous surface. One factor effecting the rate of flow is the height of the liquid added to the container. Figure 2 depicts this embodiment.

One can increase the rate of infectivity by continually adding new medium containing the vector to the system, including recycling liquid that has already flowed through and that still contains vectors. Figure 3 depicts this embodiment.

Convective flow can also proceed by applying positive pressure. In one embodiment of this method, the apparatus further comprises a pump operably attached to a reservoir holding the liquid. The apparatus container is closed and the pump pumps the liquid from the reservoir into the container under pressure. The pressure range cannot be so great as to harm the cells. Figure 4 depicts this embodiment.

Convective flow can also proceed by applying negative pressure (i.e., suction). In one embodiment of this method, the apparatus further comprises a pump operably attached to a space below the surface which supports the cells. The pump sucks liquid past the cells

and through the porous surface. Figure 5 depicts this embodiment.

Convective flow can also proceed by applying enhanced gravitational force to the liquid. In this embodiment, the apparatus further includes a centrifuge for spinning the container. The process of centrifugation causes the liquid to pass through the filtration membrane faster than it does due to gravity alone. Centrifuges adapted for spinning culture dishes holding the container include the Beckman MODEL G56[®] (Palo Alto, California) and the SORVALL RT-6000[®] by DuPont (Wilmington, Delaware). The containers usefully are spun for less than about 5 minutes at less than about 100 g. Figure 6 depicts this embodiment.

The spinning disk apparatus can be used to enhance retrovirus transport to the target cells. The apparatus includes target cells plated on the face of a spinable disk. Such disks are used in the pharmaceutical industry to measure diffusion of compounds into a liquid. See, e.g., Cussler, 1984, *supra*, pages 75-78. The step involves spinning the disk around an axis of rotation essentially perpendicular to the face of the disk, and directing a stream of liquid medium containing the vector toward the cells and substantially along the axis of rotation. When the medium hits the cells at the center of the disk, it spins out over the surface of the disk and past the target cells. Figure 7 depicts this embodiment.

The rate of viral transport to the cell surface will increase with the square root of the angular velocity. Thus, the higher the rotation rate the more viruses that reach the target cells. The maximum rotation speed will be limited, however by the shear sensitivity of the target cells. The shear sensitivity

of different types of cell varies greatly. Thus, the rotation rate and the flow velocity of the approaching fluid have to be such that the target cells are not damaged. The shear rate experienced by the target cells
5 is expected to be below about 200 per second.

This invention is also directed to a method of causing the vectors to move towards the cells by applying electrostatic force. Viruses bear a net negative charge and can be moved by electrodifusion. In this
10 embodiment, the apparatus includes an electrophoresis unit having first and second chambers separated by an ion-permeable membrane. The first chamber contains an electrolytic fluid and a negative electrode in contact therewith. The second chamber contains an electrolytic
15 fluid and a positive electrode in contact therewith. The apparatus also contains means for applying voltage across the positive and negative electrodes. The apparatus contains negatively charged vectors and target cells supported by the ion-permeable membrane in the first
20 chamber. Figure 8 depicts this embodiment.

Cells are seeded at low density on the ion-permeable membrane. However, this density can be greater than the density used in the flow-through methods, since it does not involve flow of liquid through a porous
25 surface. Electrodifusion is achieved by applying a voltage across the positive and negative electrodes of between about 1.5-10 volts for less than about 2 hours. While increased voltage will increase the velocity of the vectors towards the cells, the amount of increased
30 voltage is limited by the damage that current causes to the vector and to the cell. Voltage can be applied intermittently or periodically to improve cell viability.

This invention is also directed to methods of causing the vectors to move towards the target cells by

centrifugal force. Most retroviruses have a diameter of about 0.1 micron. Solutions containing them are essentially colloidal and the effective settling velocity of the virus is negligible compared with diffusion in a normal gravitational field. However, centrifugation of the medium at up to 1000 g will cause the virus to settle out at rates that exceed depletion by viral decay close to the cell bed. For example, 100 g will generate a sedimentation velocity of 0.015 cm/hr, approximately the minimum to overcome diffusion. According to this embodiment, the apparatus includes a centrifuge. The cells in the centrifuge are supported on a surface at the bottom of the gravitational field so that the vectors settle out of the medium onto the cells. Figure 9 depicts this embodiment.

Cells are seeded onto the surface of multiwell plates at 5% to 100% confluency (and preferably above 50%) with the infection medium. Then the plates are centrifuged in, for example, a Beckman MODEL G56[®] (Palo Alto, California) or a Sorvall RT-6000[®] (Du Pont, Wilmington, Delaware) centrifuge at between about 100 g and about 1000 g for less than about 2 hours. After the infection period, the cells can be re-plated at lower confluency (e.g. less than 50 %) and incubated under normal growth conditions so that they can grow to confluence.

EXAMPLE I

Diffusion of Virions in Solution

Data presented in this example on infection as a function of time and volume for the same concentration virus solution indicate that there is no gain in number of infected colonies with increasing amounts of virus applied to an adherent target cell bed. This finding

shows that virus infection is limited by diffusion in the current method of infection (overlaying the target cells with a stagnant layer of infection solution).

Four liquid depths: 520 μm (500 μL), 832
5 microns (800 μL), and 1558 microns (1500 μL) were used to infect target cells seeded in six-well plates (surface area 9.62 cm^2) over 30 hours. The target cells were seeded in triplicate approximately 26 hours prior to each infection at 30,000 cells/well. Eighteen 6-well plates
10 were used. Duplicate cultures were also sacrificed for cell counts over 2-16 hours. Cell samples were obtained by washing cultures twice with 2 mL HBSS (Hanks Balanced Salt Solution) followed by incubation with 0.5 mL trypsin-EDTA. Cells were collected after they were
15 rounded up and detached from the plates, and then they were counted on the hemocytometer.

The infection solution was obtained from 5 cultures of 60%-90% confluent $\psi\text{CRIP/pMFG}$ at cell passage #17. Cells were grown in Dulbecco's Modified Eagle's
20 Medium (DMEM) (GIBCO, cat. no. 430-2800EB) supplemented with 10% fetal bovine serum (FBS) (GIBCO, cat. no. 240-6000AJ) or 10% calf serum supplemented (CSS) (GIBCO, cat. no. 200-6160AJ) to 50% to 60% of confluence before the medium was totally replaced by fresh medium. Ten Petri
25 dishes were "boosted" with fresh medium 24 hours previous, passed through conditioned 0.45 μ filters, and diluted approximately by half with fresh 10% CSS in DMEM. Polybrene was added to 4 $\mu\text{g/mL}$. Cells were overlaid with infection solution within 1.5 hours after supernatant was
30 collected. Cell counts were performed in parallel wells to ensure that the cell growth was similar at all depths.

Infection was stopped by removing infection medium and adding 2 mL fresh medium after the incubation
35 time. This process took less than seven minutes for each

sample. Cultures were stained 4-5 doublings after infection, allowing plenty of time for expression of the integrated gene. Integration has been reported to take place 0.3 to 3 days post-infection. D.R. Lowy, "Transformation and Oncogenesis: Retroviruses" in Virology, B.N. Fields et al., eds., Raven Press New York (1985).

The rounded average number of blue colonies in three trials as a function of the time the cells were exposed to infection medium are given in Table I below.

TABLE I

		Average Number of Colonies		
Infection Time (hrs)		Liquid depth 520 microns	Liquid depth 832 microns	Liquid depth 1558 microns
0		0.7	0.3	0.3
2		43	52	35
4		56	48	49
6		68	69	81
8	15	72	63	98
11		110	99	96
14		129	96	93
18		139	103	117
24		102	97	104
28	25	98	94	137
33		129	129	129

These results show no increased infection with increasing liquid depth. Thus, the viruses that are added to the wells but are located at a distance exceeding about 520 microns do not reach the target cells in significant numbers and thus infection is not increased. Therefore only the viruses that are located close to the cell bed initially can reach the target cells and cause infection before they are inactivated.

The process of retroviral infection is thus diffusion limited.

EXAMPLE II

Passing Virions Through a Porous Surface

5 Flowing infection solution past a cell bed dramatically increased the rate of infection compared to cultures with static fluid.

 In a first experiment, 1.5 mL and 4 mL of a virus-containing supernatant were added to a 0.4 micron
10 collagen membrane inserts (TRANSWELL®, COSTAR) seeded with CV-1 target cells one day previous at 30,000 cells per TRANSWELL® insert. These TRANSWELL® inserts were suspended separately above an aseptic vial that collected the fluid that flowed through the membrane. Each insert
15 was filled with fluid and flow was induced by gravity. 1.5 mL was also added to a seeded insert with an additional 3.2 mL in the well below so that the liquid levels remained equal, and thus no flow would be induced (static culture reference point).

20 After 8.5 hours of exposure, 0.35 mL remained in the insert from 1.5 mL originally added (about 1.15 mL flowed through), and 1.4 mL remained from the 4 mL originally added (about 2.6 mL flowed through). The static culture control contained about 1.5 mL. Hank's
25 balanced salt solution (HBSS) was used to wash the inserts before fresh growth medium was added. The extent of infection was determined by counting the number of blue cells as described in Example I.

 The results are shown in Table II. This
30 presents the relative infectivities as a function of volume of liquid flowed through. Because different

heights of liquid were used, these results represent flow rates of about 0.1 cm/hr to 0.2 cm/hr.

TABLE II

	flow (ml)	fraction blue cells	relative infectivities (to static cultures)
5	0.000	0.014	1.000
	1.500	0.100	7.143
	3.000	0.260	18.571
	3.900	0.400	28.571
10	4.500	0.380	27.143

Thus, with modest flow of infection medium through the cell bed almost 30-fold increase in the number of infected cells was observed.

In a second experiment, a range of infection solution volumes (1-6 mL) was added to seeded inserts suspended above aseptic vials, as described above. Static cultures containing 2 mL infection solution in the insert and approximately 3 mL in the wells were used as controls. Virus-containing supernatants were used in the static cultures and on the suspended inserts. Approximately 1-6 mL infection solution flowed through the inserts and no effective volume flowed through the static control during the 9 hour infection period. Cultures were treated as in the prior experiment after this point.

The results are shown in Table III. Again, because different heights of liquid were used, these results represent flow rates of between about 0.1 cm/hr and about 0.2 cm/hr.

TABLE III

	flow (ml)	fraction blue cells	relative infection rate (to static cultures)
	0.000	0.032	1.000
5	1.000	0.083	2.594
	1.000	0.102	3.188
	2.000	0.204	6.375
	2.000	0.222	6.938
	2.300	0.241	7.531
10	2.400	0.337	10.531
	4.000	0.436	13.625
	4.000	0.401	12.531
	5.000	0.552	17.250
	5.000	0.394	12.312
15	6.000	0.523	16.344
	6.000	0.515	16.094

In this experiment about 16 to 17 fold increase in infection rate was observed. The difference between these results and the previous experiment are probably
 20 due to differences in viral titer.

In a third experiment, NIH/3T3, a mouse fibroblast cell line (ATCC CRL 1658) was used as target. 3T3 cells were cultivated in the same manner as the CV-1 cells described in Example I and seeded onto TRANSWELL
 25 collagen inserts at 15,000 cells/24.5 mm insert one day previous to infection. Virus containing medium was prepared and collected as described in Example I. No dilution of the virus medium was used in this experiment and no polybrene was added. Gravity flow infections took
 30 place up to ~10 hours at ~1 mL/hour, in the same manner as described earlier (in this example). After each infection point (as indicated by the volume flowed through the inserts), the virus containing medium was removed from the insert and replaced with fresh growth
 35 medium (described in Example I). Cultures were allowed to grow for 3 days before being assayed for percentage

infection by flow cytometry (described below). The relative infections shown in Table IV are based on the average stagnant infection that occurred after 9.75 hours of exposure to virus solution.

5 Flow Cytometry: Each insert was washed three times with 2 mL Hank's buffered saline solution (HBSS) and the cells removed by 1 mL trypsin (Gibco) exposure. Reagents from the FluoReporter lacZ gene detection kit from Molecular Probes (Eugene, OR) were used to prepare and
10 stain the cells. The trypsinized cells were resuspended in microcentrifuge tubes with growth medium and put in a 37°C water bath for 5 minutes. The cells were loaded with substrate by hypotonic shock as follows: 50 μ l of 2 mM fluorescein di-b-D-galactopyranoside (FDG) was added
15 to each tube at 37°C and left to incubate for 90 seconds. The tubes were then plunged into ice, and 500 μ l ice cold PBS with human IgG (Sigma) and 1 mg/ml propidium iodide (PI) was added. A Coulter EPICS flow cytometer was used to gate single cells from a 90LS-FALS 2D dot plot, and
20 percentage gated cells lying above log mean channel number 110 was measured.

Channel number 110 was chosen as a conservative indicator of positive green (fluorescein) fluorescence from the bimodal histograms. The two peak locations did
25 not vary significantly between the bimodal histograms. Autofluorescence (no FDG staining) and the negative controls (flow with only growth medium) consistently showed distinct peaks at channel numbers far below the positive signal gate (channel number 110). All samples
30 were assayed within six hours after staining. Duplicate samples (identical volumes of infection solution were placed in the inserts for infection) were run in the latter half of this period, and early samples were rerun at the end. No significant difference in percent
35 infections were measured as a function of time of assay.

Percent infections were calculated by dividing the area in the positive fluorescence peak by the total histogram area.

TABLE IV

	Volume Flowed (mL)	% Infection	Relative Infections (based on stagnant cultures)	
5	0	0.29	0.74	
	0	0.41	1.05	
	0	0.46	1.26	
	2.8	8.98	23.03	
	2.8	11.83	30.33	
10	3.2	8.25	21.15	
	3.5	15.56	39.90	
	4.9	18.01	46.41	
	2.6	20.38	52.26	
	9.7	35.44	90.87	
15	10.9	34.97	89.67	
	6.7	34.43	88.28	
	8.5	44.54	114.21	
	6.8	32.09	82.28	
	7.6	33.46	85.79	
20	10.7	42.02	107.74	
	10.3	36.35	93.21	
	11.2	32.53	83.41	
	AVERAGE	AVERAGE	Relative Infections	
25	Volumed Flowed (mL)	%Infection	(based on stagnant cultures)	stndev
	0.00	0.39	1.02	0.261597655
	2.93	9.69	24.84	4.850583604
	3.67	18.01	46.19	6.182483226
	9.10	34.95	89.61	1.295908022
30	7.63	36.70	94.09	17.50507268
	10.73	36.97	94.79	12.24348458

23A

Although the invention has been described with reference to the presently-preferred embodiment, it should be understood that various modifications can be
5 made without departing from the spirit of the invention.

Accordingly, the invention is limited only by the following claims.

We claim:

1. A method of increasing the frequency of contact between vectors and stationary target cells in an apparatus containing them, comprising the step of causing the vectors to move towards the target cells.

2. The method of claim 1 wherein the vectors are contained in a liquid and the step comprises flowing the liquid past the target cells.

3. The method of claim 2 wherein the apparatus comprises a container having a porous surface which supports the target cells, wherein the container contains the liquid in contact with the porous surface, and wherein the step comprises moving the liquid through the porous surface.

4. The method of claim 3 wherein the step of moving the liquid comprises allowing the liquid to move at a rate of about 0.01 cm per hour to about 1.0 cm per hour.

5. The method of claim 4 comprising moving the liquid past the target cells for about 1 hr to about 10 hours.

6. The method of claim 3 wherein the step comprises allowing the liquid to move under the force of gravity.

7. The method of claim 3 wherein the apparatus further comprises a pump operatively attached to a reservoir holding the fluid and to the container, and the step comprises pumping the liquid from the reservoir into the container.

8. The method of claim 3 wherein the apparatus further comprises a pump operatively attached to a space below the surface which supports the cells, and the step comprises applying suction to the liquid.

5 9. The method of claim 3 wherein the apparatus further comprises a centrifuge for spinning the container, and the method comprises allowing the liquid to move through the porous surface under the force of enhanced gravity created by spinning the container in the
10 centrifuge.

10. The method of claim 9 wherein spinning proceeds for less than about 5 minutes at below about 100 g.

11. The method of claim 1 wherein the
15 apparatus comprises target cells plated on the face of a spinable disk and wherein the vectors are caused to move towards the target cells by spinning the disk around an axis of rotation essentially perpendicular to the face of the disk, and by directing a stream of liquid containing
20 the vectors toward the cells and substantially along the axis of rotation.

12. The method of claim 3 wherein the vectors are viruses, spheroplasts or liposomes containing genes, and free nucleic acids containing genes.

25 13. The method of claim 12 wherein the vectors are viruses having a half-life less than 24 hours.

14. The method of claim 12 wherein the vectors are retroviruses.

15. The method of claim 14 wherein the retroviruses include within their target range hematopoietic stem cells.

16. The method of claim 15 wherein target cells are human hematopoietic stem cells.

17. The method of claim 1 comprising causing the vectors to move in the direction of the cells by applying electrostatic force to the vectors.

18. The method of claim 17 wherein the apparatus comprises

(a) an electrophoresis unit having first and second chambers separated by an ion-permeable membrane, the first chamber comprising an electrolytic fluid and a negative electrode in contact therewith, the second chamber comprising an electrolytic liquid and a positive electrode in contact therewith; and

(b) means for applying voltage across the positive and negative electrodes, wherein the vectors are contained in the first chamber and the ion-permeable membrane supports the target cells in the first chamber.

19. The method of claim 18 wherein the force is about 1.5V to about 10V.

20. The method of claim 1 comprising causing the vectors to move towards the target cells by centrifugal force.

21. The method of claim 20 wherein the apparatus comprises a centrifuge having a rotor which has a surface which supports the target cells under

centrifugal force and a liquid containing the vectors in contact with the surface.

22. The method of claim 21 wherein the centrifugal force is between about 100 g and about 1000
5 g.

23. The method of claim 22 wherein centrifugal force is applied for less than 2 hours.

24. The method of claim 1 further comprising the step of culturing the cells, after infection, under
10 conditions to allow cell growth.

25. An apparatus comprising vectors contained in a liquid, stationary target cells and means for causing the liquid to move towards the target cells.

26. The apparatus of claim 25 wherein the
15 means for moving the liquid comprises a container which has a porous surface capable of allowing passage of the liquid through it, and which supports the target cells.

27. The apparatus of claim 26 wherein the porous surface comprises a filtration membrane having
20 pore sizes between about 0.1 microns about 2 microns.

28. The apparatus of claim 27 wherein the container is a cell culture insert.

29. The apparatus of claim 28 further comprising means for allowing the liquid to move under
25 the force of gravity.

30. The apparatus of claim 27 further comprising a pump operatively attached to a reservoir holding the fluid and to the container, the pump capable

of pumping the liquid from the reservoir into the container.

31. The apparatus of claim 27 further
5 comprising a pump operatively attached to a space below the surface which supports the cells, the pump capable of applying suction to the liquid.

32. The apparatus of claim 26 wherein the
10 vectors are viruses, spheroplasts comprising nucleic acid molecules, liposomes comprising nucleic acid molecules, plasmids or nucleic acid fragments.

33. The apparatus of claim 26 wherein the
vectors are viruses having a half-life less than 24 hours.

15 34. The apparatus of claim 26 wherein the
vectors are retroviruses.

35. The apparatus of claim 34 wherein the
retroviruses include within their target range
hematopoietic stem cells.

20 36. The apparatus of claim 35 wherein target
cells are human hematopoietic stem cells.

37. The apparatus of claim 26 wherein the
cells are seeded at about 5% to about 50% of confluence
on the porous surface.

25 38. An apparatus comprising (a) an
electrophoresis unit having first and second chambers
separated by an ion-permeable membrane, the first chamber
comprising an electrolytic fluid and a negative electrode
in contact therewith, the second chamber comprising an
30 electrolytic liquid and a positive electrode in contact

therewith; and (b) means for applying voltage across the positive and negative electrodes; and further comprising negatively charged vectors and target cells supported by the ion-permeable membrane in the first chamber.

- 5 39. An apparatus comprising a centrifuge having a container that has a porous surface that supports stationary target cells upon spinning the container, wherein the container contains a liquid having vectors in contact with the surface, and wherein the
10 surface of allows passage of the liquid through it, upon spinning the container.

40. The apparatus of claim 26 wherein the porous surface comprises a filtration membrane having pore sizes between about 0.1 microns about 2 microns.

- 15 41. An apparatus comprising target cells plated on the face of a spinnable disk, a liquid comprising vectors, means for spinning the disk around an axis of rotation essentially perpendicular to the face of the disk and means for directing a stream of the liquid
20 towards the target cells and substantially along the axis of rotation.

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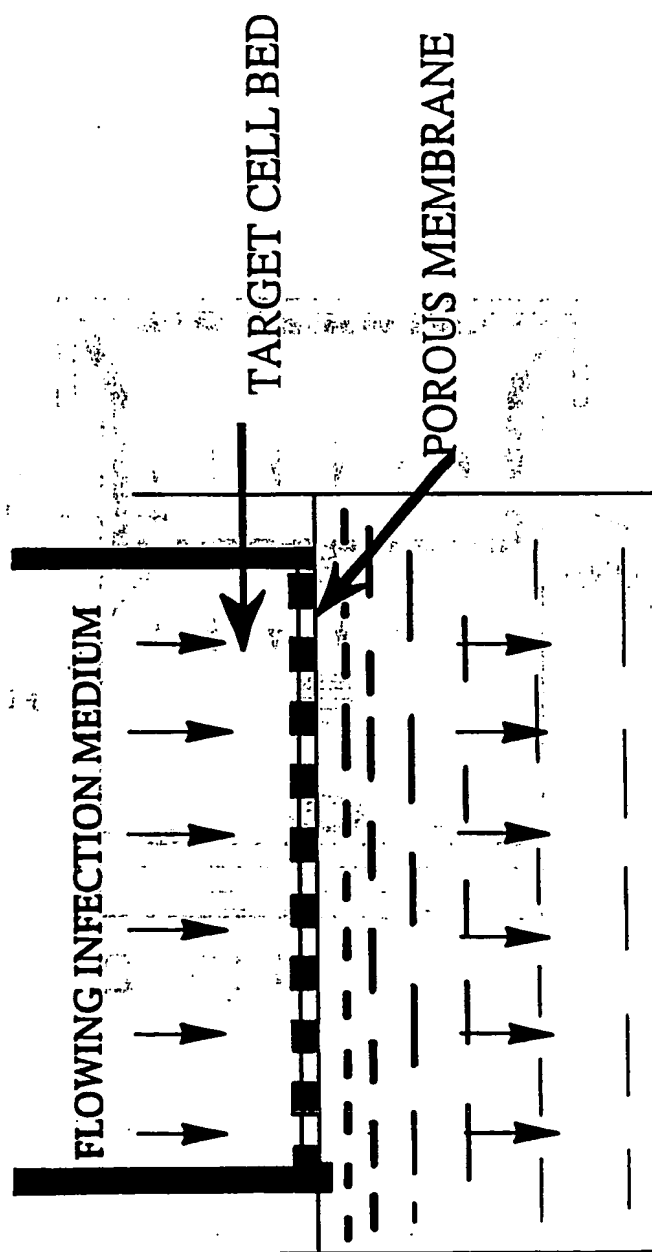


FIG. 1

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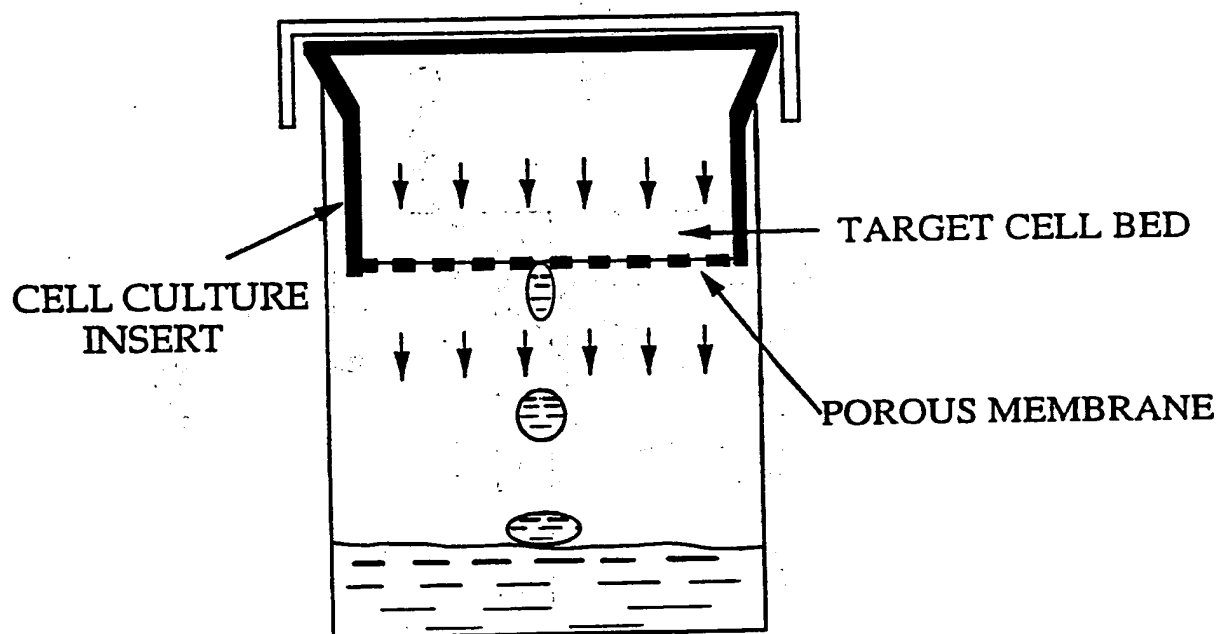


FIG. 2

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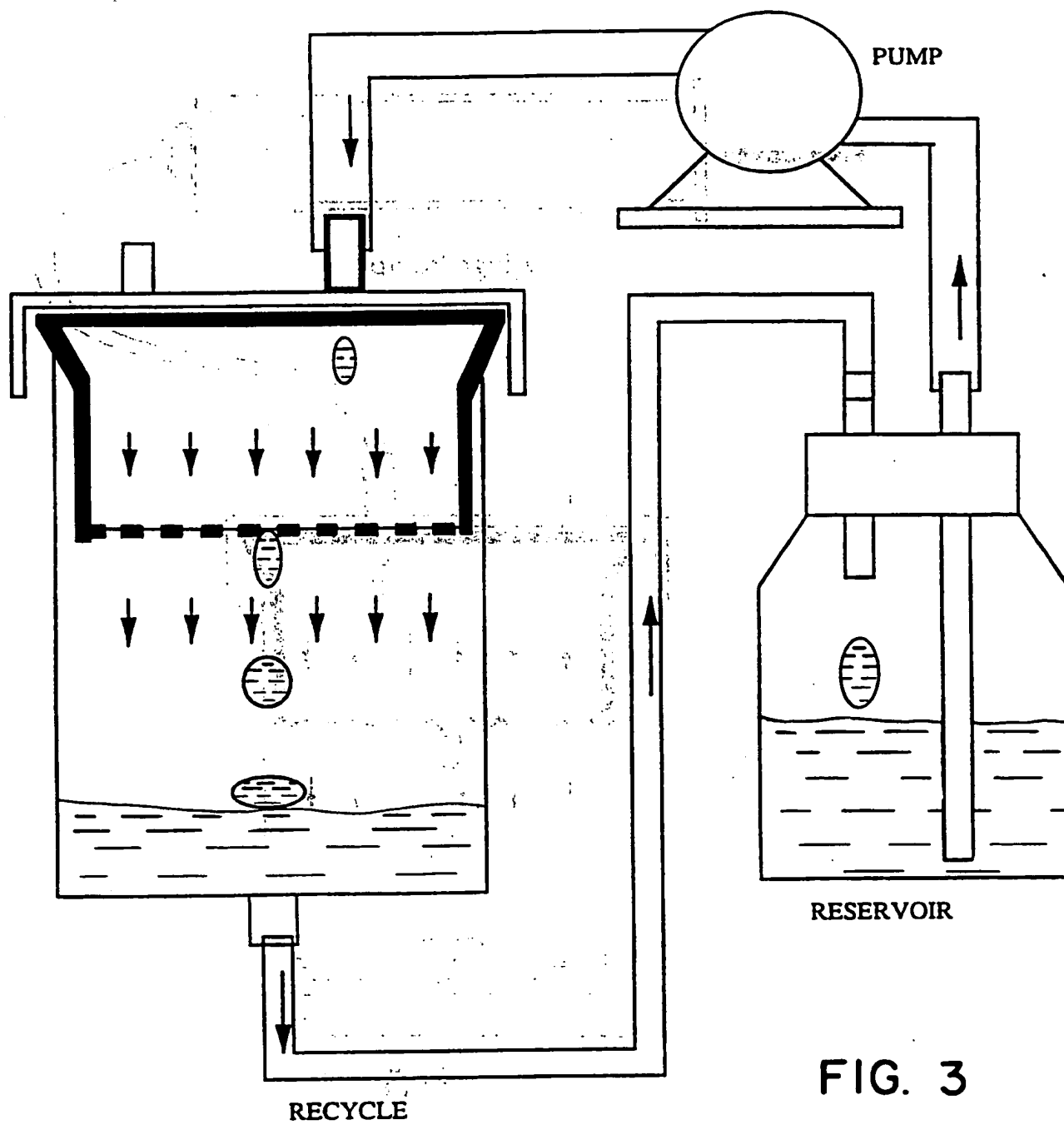


FIG. 3

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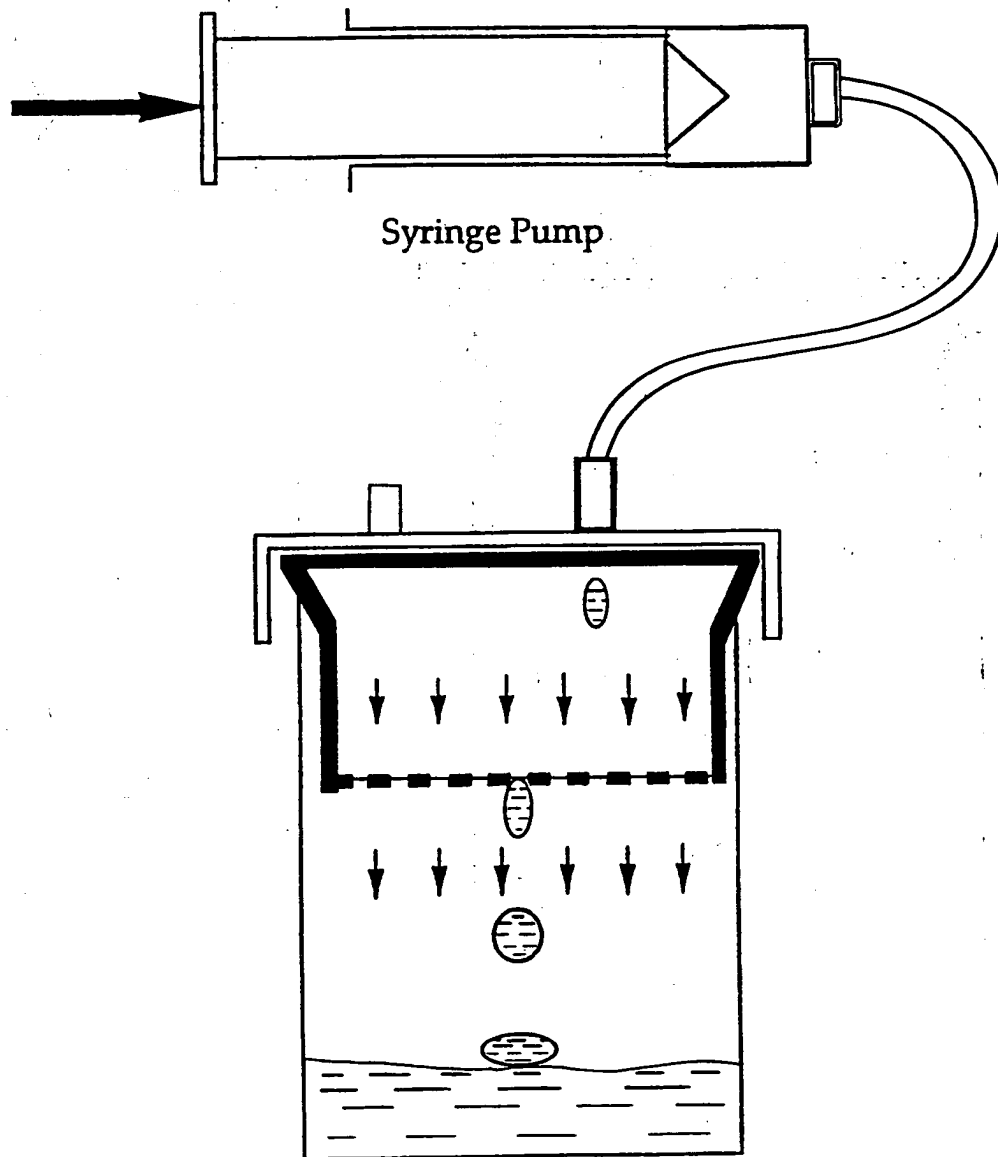


FIG. 4

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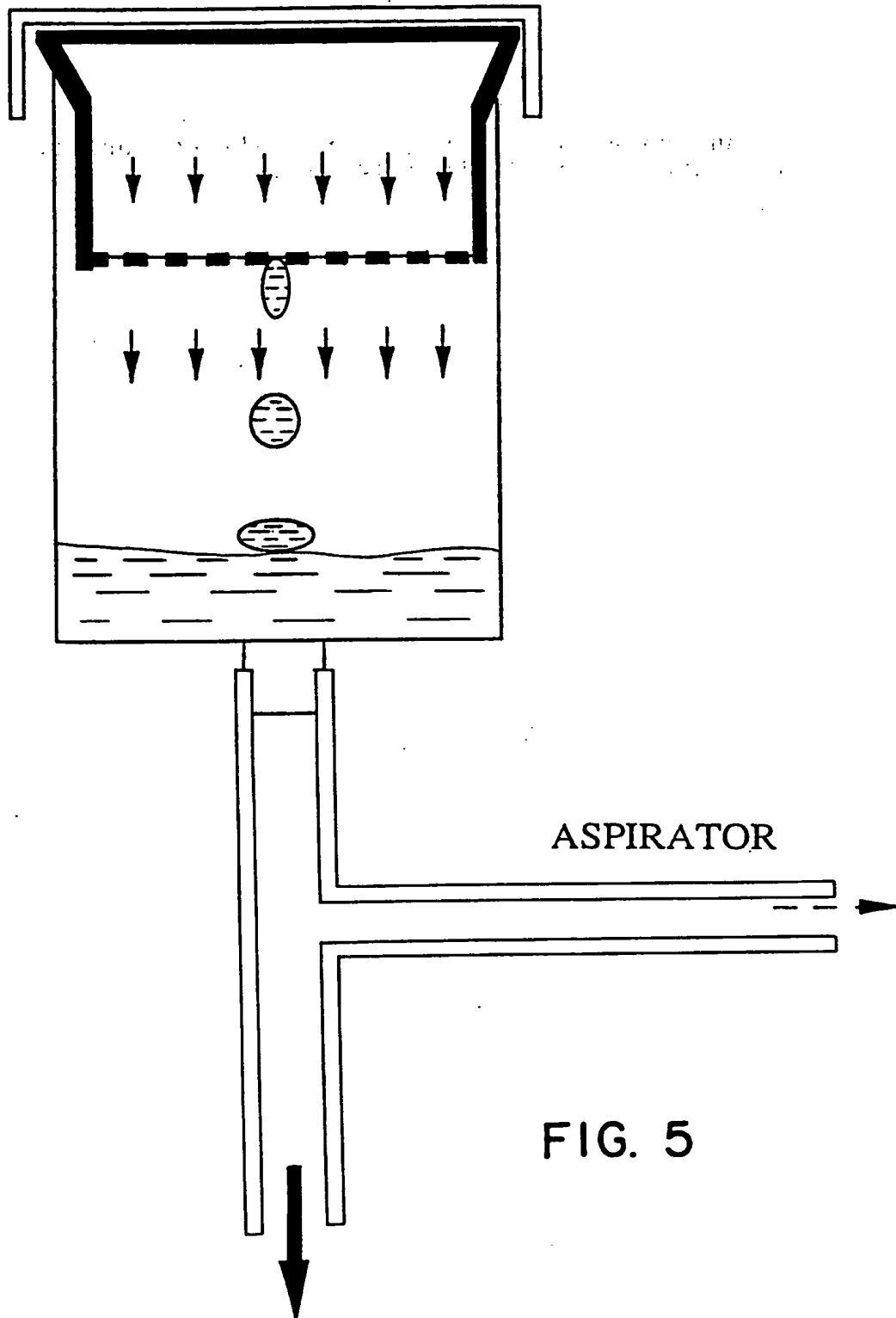


FIG. 5

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Any reference to Figure 6 shall be considered non-existent (see Article 14(2)).

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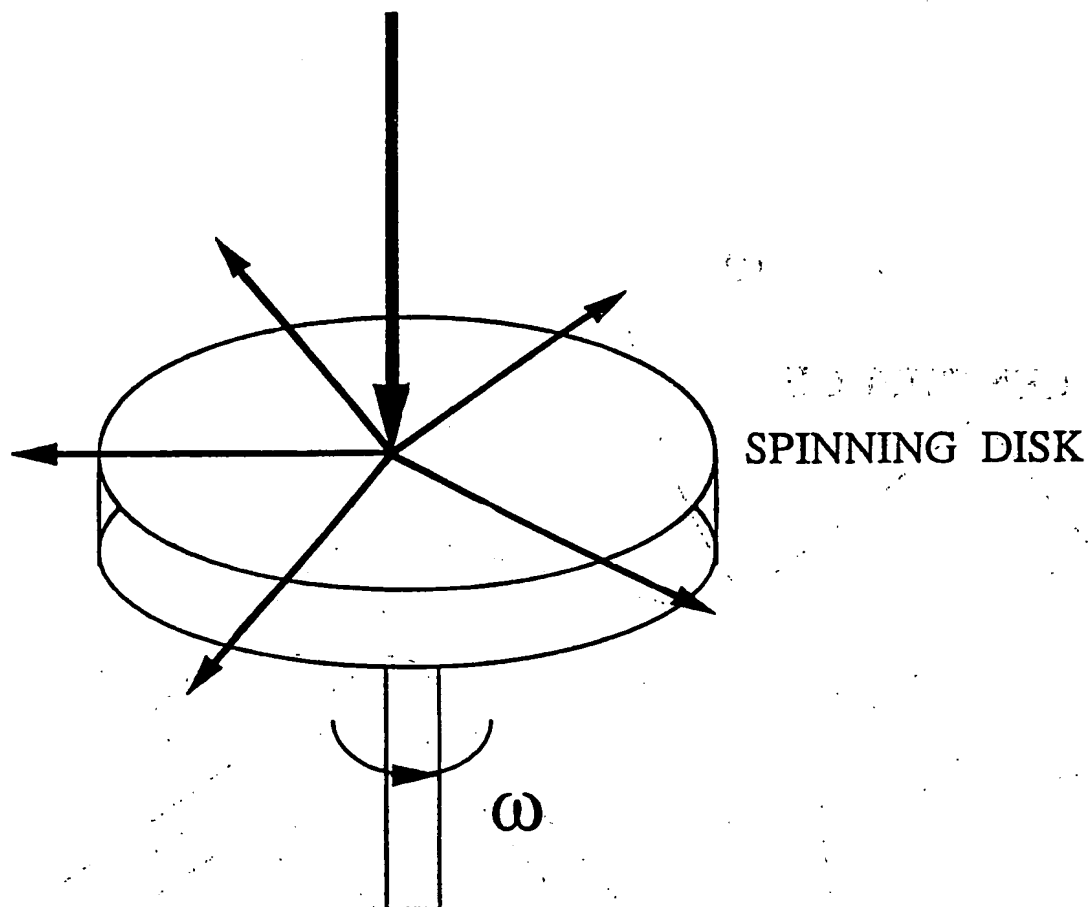


FIG. 7

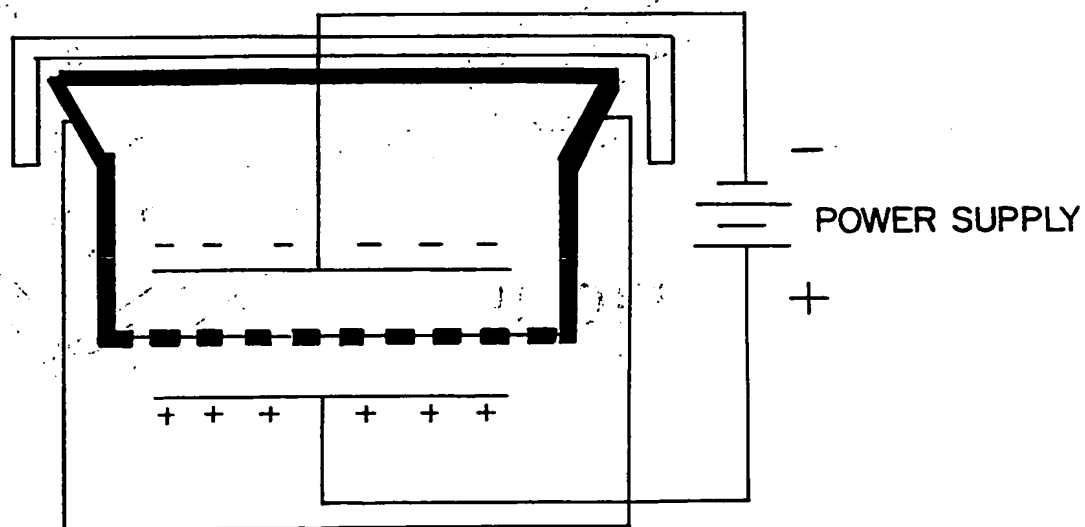
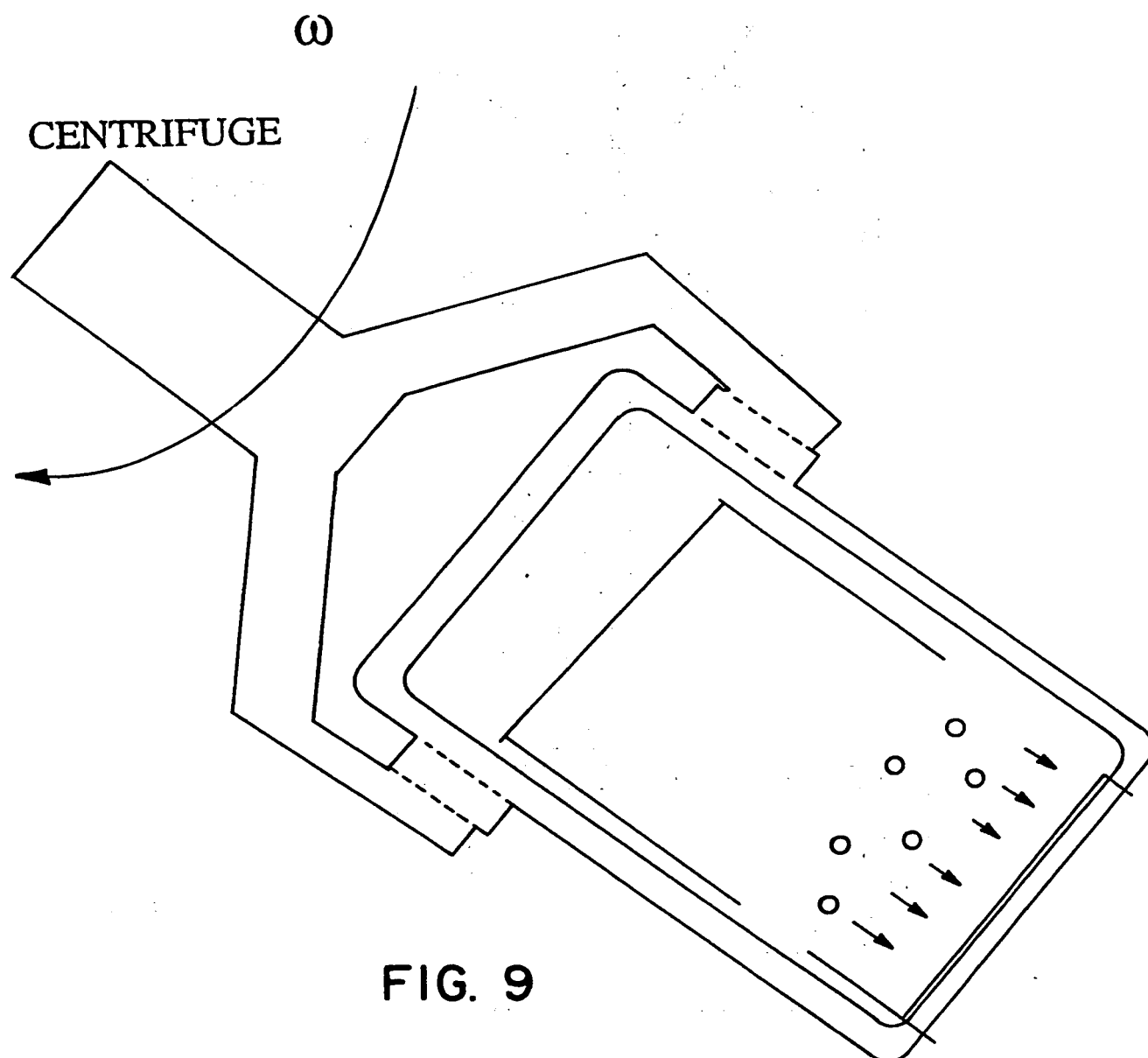


FIG. 8

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(21) International Application Number: PCT/US94/11285 (22) International Filing Date: 6 October 1994 (06.10.94) (30) Priority Data: 08/134,105 8 October 1993 (08.10.93) US (71) Applicant: THE UNIVERSITY OF MICHIGAN [US/US]; 3003 South State Street, Woverine Tower, RM 2071, Ann Arbor, MI 48109 (US). (72) Inventors: PALSSON, Bernhard, O.; 345 Meadow Creek Drive, Ann Arbor, MI 48105 (US). CLARKE, Michael, F.; 3377 Craig Road, Ann Arbor, MI 48103 (US). CHUCK, Alice, S., Y.; 2222 Fuller Road #607A, Ann Arbor, MI 48105 (US). (74) Agents: CAMPBELL, Cathryn et al.; Campbell & Flores, Suite 700, 4370 La Jolla Village Drive, San Diego, CA 92122 (US).			(81) Designated States: AM, AU, BB, BG, BR, BY, CA, CN, CZ, FI, GE, HU, JP, KG, KP, KR, KZ, LK, LT, LV, MD, MG, MN, MW, NO, NZ, PL, RO, RU, SI, SK, TJ, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i> (88) Date of publication of the international search report: 13 July 1995 (13.07.95)

(54) Title: METHODS OF INCREASING RATES OF INFECTION BY DIRECTING MOTION OF VECTORS**(57) Abstract**

This invention provides methods of increasing the frequency of contact between vectors and stationary target cells in an apparatus containing them which involves causing the vectors to move towards the target cells with motion above and beyond random Brownian motion. The methods of this invention include causing the vectors to move in the direction of the cells by (1) causing flow-through of a liquid containing the vectors through or past a cell bed, (2) moving charged vectors towards the target cells by electrodiffusion and (3) centrifuging vectors and cells to cause settling of vectors onto the cells.

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INTERNATIONAL SEARCH REPORT

Intern: al Application No
PCT/US 94/11285

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/67 C12N15/87 C12M1/10 C12M1/42 C12M3/06
C12N15/63

According to International Patent Classification (IPC) or to both national classification and IPC

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IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE WPI Section Ch, Week 9403, Derwent Publications Ltd., London, GB; Class B04, AN 94-021918 & JP,A,5 328 966 (RIKAGAKU KENKYUSHO) 14 December 1993 see abstract *see the whole abstract*</p>	1, 17-19, 38
X	<p>DATABASE WPI Section Ch, Week 9132, Derwent Publications Ltd., London, GB; Class B04, AN 91-234068 & JP,A,3 151 883 (CANON KK) 28 June 1991 see abstract see the whole abstract*</p>	1, 3, 10, 20-23, 39
-/--		

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

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Date of the actual completion of the international search

3 February 1995

Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

International Application No.
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol.57, no.4, 1991 pages 1194 - 1201 GARY M. DUNNY ET AL. 'Improved electroporation and cloning vector system for Gram-positive bacteria' *see the whole document*</p> <p>---</p>	<p>1, 17, 18, 38</p>
A	<p>PNAS, vol.89, 1992 pages 9196 - 9200 J. GAERTIG ET AL. 'Efficient mass transformation of Tetrahymena thermophila by electroporation of conjugants' *see the whole document*</p> <p>-----</p>	<p>1, 17, 18, 38</p>

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 94/ 11285

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
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Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. Claims: 1-8, 12-16, 24-37: force of gravity
2. Claims: 1-5, 9, 10, 12-16, 20-23, 39, 40: centrifugal force
3. Claims: 1-5, 11-16, 41: spinable disk
4. Claims: 1-5, 17-19, 38: electrophoresis

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-5(partially), 5-8, 12-16(partially), 24-37

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
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